



# KONGERIKET NORGE

The Kingdom of Norway

#2  
NO 00 / 00200

REC'D 30 JUN 2000
WIPO PCT

4

No 00/200

## Bekreftelse på patentsøknad nr

*Certification of patent application no*

**1999 2786**

**PRIORITY DOCUMENT**  
SUBMITTED OR TRANSMITTED IN  
COMPLIANCE WITH  
RULE 17.1(a) OR (b)

- Det bekreftes herved at vedheftede dokument er nøyaktig utskrift/kopi av ovennevnte søknad, som opprinnelig inngitt 1999.06.08

*It is hereby certified that the annexed document is a true copy of the above-mentioned application, as originally filed on 1999.06.08*

2000.06.13

*Freddy Strømmen*

Freddy Strømmen  
Seksjonsleder

*Eva Helværen*

for  
Ellen B. Olsen



**PATENTSTYRET**  
Styret for det industrielle rettsvern

N.p. søknad nr.

PATENTSTYRET

08.JUN99 992786

1c  
NORWAY  
Case 6

JOH

Søker: UNIFOB, Stiftelsen Universitetsforskning  
Bergen  
Prof. Keysersgt. 8  
5007 Bergen

Fullmektig: A/S Bergen Patentkontor  
C.Sundtsgr.36  
5004 Bergen.

Oppfinner: Geir Olve Skeie  
Gørbitsgt. 1  
5035 Bergen og

Francesco Zonzato  
Via Vittoria 21  
44100 Ferrara  
Italy

Prioritet fra: Ingemar

08.06.99

Detection of ryanodine receptor antibodies.

The present invention relates to methods, kits and compositions for the detection of ryanodine receptor antibodies in patient serum samples. The invention also 5 relates to a method for the manufacture of a pharmaceutical agent for the prevention and/or treatment of the disease myasthenia gravis, and a method of myasthenia gravis prognosis.

Myasthenia gravis (MG) is a disease of the 10 neuromuscular junction caused and characterised by antibodies against the Acetyl Choline Receptor (AChR) of the muscle endplate. The AChR antibodies cause a complement mediated damage to the postsynaptic part of the muscle endplate leading to impaired neuromuscular transmission, 15 muscular weakness and fatiguability (Lindstrom, J., D. Schelton, and Y. Fujii. 1988, *Adv. Immunol.* 42: 233-284). However, some MG patients, mainly with thymoma, have antibodies also against other muscle antigens.

Electron microscopic studies have shown that MG sera 20 binds to an unidentified protein in sacroplasmic reticulum (SR) membranes (Mendell, J.R., J.N. Whitaker, and W.K. Engel. 1973, *J. Immunol.* 111: 847-856; Flood, P.R., R. Bjugn, N.E. Gilhus, H. Hofstad, R. Matre, and J.A. Aarli. 1987, *Ann. N.Y. Acad. Sci.* 505: 732-734). Mygland et al., 25 showed that MG sera from thymoma patients stained a high molecular weight protein in a SR preparation (Mygland, Å., O.B. Tysnes, J.A. Aarli, P.R. Flood, and N.E. Gilhus. 1992,

J. Neuroimmunol. 37: 1-7), which was later identified to be identical to the  $\text{Ca}^{2+}$  release channel of SR, i.e. the ryanodine receptor (RyR) (Mygland, Å., O.B. Tysnes, R. Matre, P. Volpe, J.A. Aarli, and N.E. Gilhus. 1992, Ann.

5 Neurol. 32: 589-591).

The RyR is a  $\text{Ca}^{2+}$ -release channel located in the SR of striated muscle. It plays an essential role in muscle contraction by responding to sarcolemma depolarisation with the opening of the ion channel and the release of  $\text{Ca}^{2+}$  from 10 SR to contractile proteins in the myoplasm (Coronado, R., J. Morrissette, M. Sukhareva, and D.M. Vaughan. 1994, Am. J. Physiol. 266: c1485-c1504).

RyR antibodies were found in approximately half of thymoma MG patients but not in non-thymoma late-onset MG, 15 early onset MG, blood-donors or patients with other autoimmune diseases (Mygland, Å., O.B. Tysnes, R. Matre, P. Volpe, J.A. Aarli, and N.E. Gilhus. 1992, Ann. Neurol.

32: 589-591). The RyR antibodies are mainly of the IgG1 and IgG3 subclasses (Mygland, Å., O.B. Tysnes, J.A. Aarli, R. Matre, and N.E. Gilhus. 1993, J. Autoimmunity 6: 507-515), and stain both the skeletal and cardiac form of the RyR (Mygland, Å., O.B. Tysnes, R. Matre, J.A. Aarli, and N.E. Gilhus. 1994, Autoimmunity 17: (4) 327-31).

The presence of RyR antibodies correlate to MG 25 severity and even death with a nice correlation also to RyR antibody levels. Thymoma MG patients with RyR antibodies have in contrast to RyR negative patients often heart disease leading to sudden cardiac arrest. Others die of respiratory failure due to the neuromuscular weakness 30 (Mygland, Å., O.B. Tysnes, R. Matre, J.A. Aarli, and N.E. Gilhus. 1994, Autoimmunity 17: (4) 327-31.; Mygland, Å., J.A. Aarli, R. Matre, and N.E. Gilhus. 1994, J. Neurol. Neurosurg. Psychiatry. 57: 843-846; Skeie, G.O., E. Bartoccioni, A. Evoli, J.A. Aarli, and N.E. Gilhus. 1996, Eur. J. Neurol. 3: 136-140).

The mechanisms leading to muscular fatiguability in MG patients might be more complex than what can be explained from the AChR antibody model alone. Pagala and others have found evidence for disordered (Pagala, M., N.V. Nandakumar, 5 S.A.T. Venkatachari, K. Ravindran, T. Namba, and D. Grob. 1990, *Muscle and Nerve* 13: 1012-1022; Pagala, M., N.V. Nandakumar, S.A.T. Venkatachari, K. Ravindran, B. Amaladevi, T. Namba, and D. Grob. 1993, *Muscle and Nerve* 16: 911-921) excitation-contraction coupling, for which the 10 RyR is essential, in individual MG patients. The MG patients RyR antibodies are able to inhibit binding of ryanodine to the RyR indicating that the RyR antibodies lock the RyR in the closed position (Skeie, G.O., P.K. Lunde, O.M. Sejersted, Å. Mygland, J.A. Aarli, and N.E. 15 Gilhus. 1998, *Muscle and Nerve* 21: 329-33). Patients with inhibiting antibodies had a more severe disease than patients without such antibodies (Skeie et al., 1998).

MG sera containing striational antibodies have been shown to inhibit caffeine induced  $\text{Ca}^{2+}$  release in rat 20 muscle cells (Asako et al., 1997). Experimental RyR antibodies can affect RyR function in vitro (Treves S, Chiozzi P, Zorzato F (1993) *Biochem J* 291, 757-763) and a rat strain which develop spontaneous thymomas and RyR antibodies have muscular weakness and fatiguability 25 resembling MG without detectable AChR antibodies (Iwasa, K., K. Komai, T. Asaka, E. Nitta, and M. Takamori, *Ann. N.Y. Acad. Sci.* 1998:841; 542-545). These studies might indicate a direct pathogenetic role for the RyR antibodies in MG, and thus RyR antibodies does not only have a 30 function as a disease marker.

In this study we have identified the main immunogenic region on the RyR for MG patients antibodies. We show that the antibodies reactive with this part of the RyR are able to inhibit  $\text{Ca}^{2+}$  release from SR vesicles in vitro, and 35 using a biosensor we were able to study the real time interaction between the RyR antibodies and the RyR fusion

protein containing the MIR for the RyR antibodies in MG sera.

The present invention relates to a method for the detection of ryanodine receptor antibodies in patient serum samples, said antibodies being associated with the disease myasthenia gravis, said method comprising the following steps:

(a) obtaining a serum sample from a patient suspected of having myasthenia gravis or being at risk for the development of said disease;

(b) contacting said serum sample with a composition of fusion proteins comprising the following sequences: SEQ ID NOS 1 or 2;

(c) detecting fusion protein - antibody complex formation, wherein said detected complexes indicate the presence of ryanodine receptor antibodies.

Further, the invention relates to the use of the fusion proteins comprising the following sequences: SEQ ID NOS 1 or 2 for the detection of RyR antibodies.

Also comprised by the invention is a diagnostic kit for the detection of ryanodine receptor antibodies in patient serum samples, said antibodies being associated with the disease myasthenia gravis, said kit comprising fusion proteins having the following sequences; SEQ ID NOS 1 or 2.

A preferred embodiment of the invention relates to a diagnostic kit, wherein the immunodetection reagent is a radiolabeled reagent.

The presence of pc2 or pc25 fusion protein antibodies is indicative of the presence of a thymoma.

The present invention also relates to a composition of fusion proteins useful for the detection of ryanodine receptor antibodies, which are associated with the disease myasthenia gravis, said proteins being selected from the group of proteins having a sequence of SEQ ID NO 1 or 2, or

a combination of said sequences, and to a method for the manufacture of a pharmaceutical agent for the prevention and/or treatment of the disease myasthenia gravis, wherein said agent are administered to a patient in need thereof,

5 in a amount sufficient to inhibit the binding of ryanodine receptor antibodies to the ryanodine receptor, said composition comprising a panel of fusion proteins having sequences SEQ ID NOS 1 and/or 2, and to a method of myasthenia gravis prognosis which involves the

10 determination of the presence of RyR antibodies wherein the RyR antibodies are identified by the use of the fusion proteins pc2 and pc25.

The invention will now be described further with respect to the following examples and the accompanying

15 drawing in which:

Figure 1A shows in diagrammatic form the Ryanodine receptor fusion proteins strategy. Figure 1B shows the induced E. coli extracts loaded in the gel. Proteins were separated by electrophoreses, blotted into nitrocellulose

20 and stained with Ponceau Red. The lower row shows the Western blot staining.

Figure 2A shows a Ryanodine receptor fusion protein strategy designed to narrow the immunopositive region. Figure 2B shows the induced E. coli extracts loaded in the

25 gel. The lower row shows the Western blot staining (stained with Ab from patients).

Figure 3 shows the fragments pc2, pc2A and pc2B blotted onto nitrocellulose membranes and stained with Ab from patient

30 Figure 4 shows the characterisation of the binding between MG patients antibodies and the pc2 fusion protein. Fig 4A shows the curves form injections with anti-path antibody in concentrations ranging from 2.5-25 mg/ml (Puickinject, flowrate 5  $\mu$ l/min in HBS, pH 7.4) in the

35 flowcell with bound pc2. The maximal response was 400 RU. Figure 4B shows the results from injections of IgG

fractions from MG patients and controls. Non covalently bound proteins were removed and the sensor chip regenerated by injections of 5  $\mu$ l of 0.5 % SDS and/or 3 M guanidinium chloride in 5 mM Tris, pH 8, between injections of serum or IgG samples. Figure 4C shows the results from the sandwich assay. The anti-patch antibody was immobilised on the sensor chip; and a) the injection of electroeluted pc2-Ry1 fusion protein gave a signal of 60 RU, and b) the second injection of Ry1 antibody positive MG IgG lead to an additional increase of 180 RU which is consistent with a Ab binding stoichiometry of 1.

Figure 5 shows 4-chloro-m-cresol (4-cmc) induced  $\text{Ca}^{2+}$  release from skeletal muscle SR.  $\text{Ca}^{2+}$  concentrations are represented by the A710-790 of the  $\text{Ca}^{2+}$  indicator antipyrolazo III. SR were added to the cuvette followed by 6 consecutive additions of 20 nmol  $\text{CaCl}_2$  to load the vesicles with  $\text{Ca}^{2+}$ . 50  $\mu\text{l}$  of IgG (0.5 mg/ml) from patients and controls were added to the cuvettes for a 2 minutes incubation, followed by addition of 4-chloro-m-cresol (200 mM) to induce  $\text{Ca}^{2+}$  release. Curve A shows a normal  $\text{Ca}^{2+}$  release with IgG from a RyR negative MG patient. Curve B shows that IgG from a Ry1 positive patient strongly inhibits  $\text{Ca}^{2+}$  release. Curve C shows a normal  $\text{Ca}^{2+}$  release after removing Ry1 antibodies from the IgG by preincubation with pc2.

Figure 6 shows the 4-chloro-m-cresol induced  $\text{Ca}^{2+}$  release form SR reacted with IgG from MG patients and controls.

Figure 7 shows the  $\text{Ca}^{2+}$  release from SR reacted with IgG fractions with and without Ry1 antibodies at different 4-Chloro-m-cresol concentrations. The Ry1 antibodies shift the curve to the right suggesting an allosteric inhibition.

Experimental sectionMethods and resultsThe sera used in the assay

5       The study included sera from 122 (75) thymoma MG patients (37 Italian, 38 Norwegian, 19 late-onset MG patients and 25 early-onset MG patients (all Norwegian) which had previously been tested in WB for RyR antibodies using a SR preparation as antigen (Mygland et al., 1992; 10 Skeie, G.O., E. Bartoccioni, A. Evoli, J.A. Aarli, and N.E. Gilhus. 1996, *Eur. J. Neurol.* 3: 136-140). The medical records were reviewed and the patients scored according to MG severity at peak of illness and at the last follow-up as previously described (Mygland et al., 1994; Skeie et al., 15 1996; Skeie, G.O., Å. Mygland, J.A. Aarli, and N.E. Gilhus. 1995, *Autoimmunity* 20: 99-105). In addition sera from 20 Norwegian blood-donors and 3 SLE patients were used as controls.

20       Biosensor and calcium release

For the biosensor and calcium release assay purified IgG fractions from patient and control sera were obtained using protein G columns according to the protocols provided by Pharmacia AB (Uppsala, Sweden). The IgG fractions were dialysed against HBS buffer and the concentrations adjusted before use to 0.5 mg/ml.

Screening of overlapping RyR fusion proteins

DNA manipulations were carried out as described in 30 Maniatis P, Fritsch EF, Sambrook J. Molecular Cloning. A panel of fusion proteins covering the entire RyR coding sequence were constructed as previously described (Treves S, Chiozzi P, Zorzato F (1993) *Biochem J* 291, 757-763; Menegazzi P, Larini F, Treves S, Guerrini R, Quadrone M, 35 Zorzato F, (1994) *Biochemistry* 33, 9078-9084). Gel electrophoresis was carried out as described by Laemmli,

U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685. Western blots of bacterial extracts were carried out overnight as described by Gershoni et al., (1985). Indirect 5 immunoenzymatic staining of Western blots was carried out as described by Young, R.A., B.R. Bloom, C.M. Grosskinsky, J. Ivanyi, D. Thomas, and R.W. Davis. 1985, *Proc. Natl. Acad. Sci. U. S. A.* 82:2583-2587), and detailed by Treves et al., (Treves S, Chiozzi P, Zorzato F (1993) *Biochem J* 10 291, 757-763).

#### RyR fusion protein Western blot

We used the pc2-RyR fusion protein as antigen (Mygland, 1992). Electrophoresis was performed on sodium 15 dodecyl sulphate (SDS) polyacrylamide gels (12%) as described by Laemmli, 1970. 200 ml pc2 (50 mg/ml) were added to 120 ml of sample buffer containing 2% (w/v) SDS, 1.5% (w/v) Tris, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue. The mixture was heated to 100° for 1 20 minute. About 100 µl of the protein mixture was applied per gel. Proteins separated on the gel were transblotted onto nitrocellulose sheets as described by Towbin, H., T. Staelin, and J. Gordon. 1979, *Proc. Natl. Acad. Sci. U.S.A.* 76: 4350-4354.

25 Nitrocellulose sheets were soaked in 5% (w/v) low fat dry milk (Nestle) in phosphate buffered saline (PBS) for 1 hour to block additional protein binding sites. They were washed 3 times in PBS with 0.05% Tween 20 (PBS-Tween), cut into vertical strips and incubated overnight at 4°C with 30 patient and control sera diluted 1:50 in PBS containing 0.5% fat free dry milk and 0.05% Tween 20 (PBS-dry milk Tween (Nestle)). After separate washings for 10 min in PBS-Tween, the nitrocellulose strips were incubated for 1 h with peroxidase-conjugated rabbit antibodies (Dako, Copenhagen, Denmark) to human IgG diluted 1:1000 in PBS dry

milk Tween. The nitrocellulose strips were then washed and developed in a peroxidase colour development solution containing 30 mg 4-chloro-1-naphthol (Sigma), 17% (v/v) cold methanol, 83% (v/v) PBS and 0.05% (v/v) H<sub>2</sub>O<sub>2</sub>. Positive  
5 and negative control sera were applied to strips from each transblotted nitrocellulose sheet.

### Results

#### Example 1

##### 10 The identification of the main immunogenic region of RyR

As shown in figures 1 and 2, MG sera reacted with two of the RyR fusion proteins examined. The reactivity was strongest with the pc2 epitope which is located near the N-  
15 terminus; residues 799-1152 and consists of 360 amino acids. The pc25 epitope is located between residues 2595-2935. The amino acid sequences (one letter codes) of the pc2 (SEQ ID NO 1) and pc25 (SEQ ID NO 2) epitopes are given below.

20 When the pc2 peptide was clipped into 2 fragments (indicated as pc2A and pc2B in figure 3) by Pst I the reactivity with the MG sera was lost indicating that the site for the restriction enzyme is located very near the binding site for the protein, or that this site is  
25 important for the conformation recognised by the MG patients antibodies.

Antibodies against pc2 were found in 57 out of 75 thymoma MG patients, 5 out of 19 late-onset MG patients, none of 25 MG hyperplasia patients and none of the 20  
30 blood-donors.

24 of the 44 thymoma MG patients examined for antibodies against pc25 in WB had antibodies reactive with this RyR epitope, but none of 20 MG hyperplasia patients and none of the 20 blood-donors. All patients positive for  
35 pc25 had pc2 antibodies while only 24 of the 33 pc2 positive patients had pc25 antibodies.

All sera with reactivity against the full length RyR in Western blots did also react with the pc2 RyR fusion protein (Table 1). The pc2 RyR fusion protein must therefore contain the main immunogenic region.

5

TABLE 1:

Number of MG patients with Ry1 antibodies using different  
10 Ry1 antigens in WB.

	Thymoma MG	Late-onset MG	Early onset MG
Ry1 antibodies 44/75 (SR)		0/19	0/25
Ry1 antibodies 57/75 (pc2)		5/19	0/25
Ry1 antibodies 24/44 (pc25)		nd	0/25

SR: sarcoplasmic reticulum; pc2: pc2 RyR fusion protein,  
pc25: pc25 Ry1 fusion protein.

15 Correlation with titin antibodies

All but 9 thymoma MG patients had titin antibodies. 13 titin positive MG patients had no pc2 RyR antibodies, while 4 patients with pc2 RyR antibodies had no titin antibodies. 5 thymoma MG patients were negative for both titin and pc2 20 RyR antibodies. 10 out of the 19 late onset sera contained titin antibodies. 5 of the sera also contained MG anti-pc2 antibodies while 5 sera with titin antibodies had no pc2 RyR antibodies. We established an ELISA (data not shown) using the pc2 fusion protein as antigen. The results must 25 be interpreted together with the Western blot data since some sera negative with the pc2 band in WB had a little back-ground staining of residual bacterial proteins which gave a low positive ELISA signal. The ELISA could therefore be used as a screening test before checking all positive

sera for reactivity with the pc2 RyR fusion protein in WB to increase the specificity.

Example 2

5   Real-time RyR antibody pc2 fusion protein interactions

The reactivity of MG sera with the pc2 fusion protein were studied using a biosensor; BIACORE 1000 (Pharmacia Biosensor AB, Uppsala, Sweden) which allows real time biospecific interaction analysis. This system uses the 10 optical phenomenon of surface plasmon resonance (SPR) which detect changes in optical properties at the surface of a thin gold film on a glass support (sensor ship) (Lofas and Johnsson, 1990). The sensorship is covered by a dextran matrix to which one reactant is covalently linked, while 15 the other(s) is introduced in a flow passing over the surface. The resonance angle depends on the refractive index in the vicinity of the surface which changes as the concentration of molecules on the surface is modified and is expressed in resonance units (RU). A signal of 1000 RU 20 corresponds approximately to a surface concentration change of 1 ng/mm<sup>2</sup>.

Immobilisation of protein to the sensor ship were done via primary amine groups using the amine coupling kit (Pharmacia Biosensor AB) according to standard procedures 25 (Lofas and Johnsson 1990; Fagerstam LG, Frostell A, Karlsson R, Kullman M, Larsson A, Malmqvist M and Butt H. (1990), J Mol Recog. 3, 208-214). The carboxylated matrix of the sensorship CM5 (Pharmacia Biosensor AB) was first activated by injection (Quickinject, flow rate 5 ml/min in 30 HBS pH 7.4 (10 Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.05% Surfactant P20 (Pharmacia Biosensor AB) of a mixture of NHS/EDS (N-hydroxy-succinimide 50 mM/N-ethyl-N-(3 diethylaminopropyl)-carbodiimide 200 mM) (50 ml). Then in one flowcell 60 ml pc2 RyR fusion protein (1.5 mg/ml in 2.5 35 mM acetate buffer, pH 4) was shown to give the best binding

in preconcentration experiments using buffers of different pH. About 2000 RU were immobilised on the sensorship.

In another flowcell about 4000 RU of an anti-path antibody were immobilised by injecting 70 ml of anti-path antibody (35 ml anti-path 0.5 mg/ml mixed with 35 ml 10 mM acetate buffer, pH 5.5). Residual activated sites were blocked by injection of 50 ml of 1 M ethanolamine hydrochloride pH 8.5 (Pharmacia Biosensor AB).

In one flowcell the sensorship was only activated by NHS/EDS and blocked by ethanolamine hydrochloride without injection of any proteins. This flow cell was used to examine non specific IgG binding to the dextrane matrix of the sensorship. 50 ml of sera diluted 1:10-1:100 in HBS buffer and purified IgG fractions (dialysed against HBS buffer) (0.5 mg/ml) from MG patients and controls were injected into the flowcells at a constant flowrate of 5 ml/ml and a sensogram recorded. Between injections the sensor chip was continuously washed with HBS buffer.

Further, different concentrations of anti-path antibody were injected into the flowcell with immobilised pc2 fusion protein to examine the amount and reactivity of the immobilised pc2 protein, and to compare the binding kinetics with the MG sera.

In the flow cell with immobilised anti-path antibodies 50 ml of pc2 fusion protein were injected prior to the injection of sera/IgG fractions. The sensor ship were regenerated by injections of 5 ml of 5% SDS and/or 3 M guanidinium chloride in 5 mM Tris pH 8 between injections of serum or IgG samples.

The curves from sensograms obtained by injecting the samples into the flow cell without proteins were subtracted from the curves obtained in flow cells with immobilised pc2 RyR fusion protein to record the specific binding results

Real time Surface Plasmon Resonance Recording

About 2000 RU of the pc2 fusion protein was immobilised on the sensorchip. When injecting different concentrations of the high affinity anti-path antibody a

5 maximal response of 400 RU was obtained. This was therefore the maximal expected response also for the patient sera. The results from injections of 50 ml of purified IgG fractions 0.5 mg/ml from MG patients and controls are shown in Figure 4B. Only IgG fractions from  
10 RyR antibody positive MG patients gave a signal above 30 RU (73-360 RU). The sera with the best binding gave a signal close to the maximal expected indicating that nearly all binding sites for the RyR antibodies were saturated.

The specificity of the interaction was also tested in  
15 a sandwich assay. When injecting 50 ml (1 mg/ml) of pc2 RyR fusion protein into the flowcell with immobilised anti-path antibody, 60 RU were bound, as shown in Figure 4C. The second injection of 50 ml RyR antibody positive MG IgG (0.5 mg/ml) gave a signal of about 180 RU (Figure 4C).

20 The molecular weight of pc2 is 60kD, and the molecular weight of the antibodies are 150 kD. As there is a 1:3 relationship between both the MW and signal in RU there is a 1:1 relationship between the RyR antibodies and the fusion protein i.e. one antibody molecule binds to one RyR  
25 fusion protein.

Example 3.Inhibition of Ca<sup>2+</sup> release

30 Ca<sup>2+</sup> measurements

SR was isolated from white muscles of New Zealand White rabbits and was fractionated into longitudinal tubules and TC in the presence of antiproteolytic agents as described by Saito, A., S. Seiler, A. Chu, and S.  
35 Fleischer. 1984, J. Cell. Biol. 99:875-885. The SR fractions were resuspended in 0.3 M sucrose, 5 mM

imidazole, pH 7.4, 100 mM PMSF, 1 mg/ml leupeptin, and were stored in liquid nitrogen until used.

Ca<sup>2+</sup> release from isolated SR fractions was measured in a Beckmann DU7400 diode array spectrophotometer by monitoring the A<sub>710</sub>-A<sub>790</sub> value of the Ca<sup>2+</sup> indicator antipyrylazo III (Fluca, Buchs, Switzerland) as described by Palade P. 1987, *J Biol. Chem.* 262: 6142-6148, and detailed by Treves S, Chiozzi P, Zorzato F (1993), *Biochem J* 291, 757-763. Pulses of 20 nM Ca<sup>2+</sup> were administered to load the SR fractions with Ca<sup>2+</sup>, and the fractions were then incubated with 50 ml of IgG (0.5 mg/ml) from patients and controls for 2 minutes before different concentrations of the Ca<sup>2+</sup> releasing agent 4-chloro-m-cresol were added. To calibrate the curves 20 nM of Ca<sup>2+</sup> were added at the end of each experiment.

IgG fractions from patients with RyR antibodies significantly inhibited 4-chloro-m-cresol induced Ca<sup>2+</sup> release from isolated SR vesicles (Figure 5). The mean Ca<sup>2+</sup> release rate was significantly lower when the SR vesicles had been incubated with IgG fractions from RyR antibodies positive MG patients ( $0.93 \pm 0.55$  mmol Ca<sup>2+</sup> per mg SR protein per min) compared with IgG fractions from RyR antibody negative MG patients ( $1.6 \pm 0.36$  mmol Ca<sup>2+</sup> per mg SR protein per min) and controls (blood-donors and SLE patients) ( $1.6 \pm 0.21$  mmol Ca<sup>2+</sup> per mg SR protein per min) ( $p=0.0021$ ). The inhibition was concentration dependent (Figure 7) and the curves fitted with a model of allosteric inhibition.

When removing the RyR antibodies by preabsorbing the IgG fractions with the pc2 fusion protein or the SR fractions for 1 hour before using them in the Ca<sup>2+</sup> release assays the inhibition of Ca<sup>2+</sup> release disappeared (Figure 5), proving that the antibodies binding to the pc2 RyR fusion protein are responsible for inhibiting Ca<sup>2+</sup> release in vitro.

Conclusions

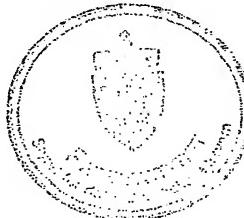
By mapping a series of overlapping RyR fusion proteins we have identified the main immunogenic region (MIR) on the 5 RyR for MG patients antibodies. All patients positive for RyR antibodies in Western blots using a SR preparation as antigen had pc2 antibodies proving that the pc2 fusion protein contains the MIR region. However, 13 patients positive for MIR antibodies was negative in the SR Western 10 blot assay, indicating that the use of the recombinant protein as antigen gives a more sensitive assay. The MIR RyR antibodies were found in 76% of thymoma MG patients but not in blood-donors or young-onset MG patients. The antibodies are therefore closely associated with thymoma MG 15 and the presence of the pc2 fusion protein antibodies strongly suggests the presence of a thymoma. The only patients without a detectable thymoma, positive for the pc2 RyR antibodies, were 5 late-onset MG patients. All of these also had titin antibodies. The late onset MG patients with 20 titin/RyR antibodies are very similar to thymoma MG patients immunologically, clinically and genetically (Aarli, J.A. 1997. Late-onset MG. *Eur. J. Neurol.* 4: 203-209). Microscopic thymomas has been described by Pescarmona, E., S. Rosati, A. Pisacane, E.A. Rendina, F. 25 Venuta, C.D. Baroni. 1992. *Histopathology*. 20: 263-266), and it is not unlikely that the late-onset MG patients with titin and RyR antibodies have a paraneoplastic MG much like the thymoma patients. Some could have a preneoplastic condition; paraneoplastic symptoms often develop years 30 before a tumour is found in a great proportions of patients in other paraneoplastic conditions (Dropcho, E.J. 1998. *Ann. N.Y. Acad. Sci.*: 841:246-261), or the thymoma could have gone into remission in a way similar to that described for other tumours (Dropcho, 1998 ). RyR antibody positive 35 MG patients should probably be thymectomized regardless of a positive CT scan. The results from the surface plasmon

resonance studies showed that there is a strong, specific one-to-one reaction between the MG patients antibodies and the pc2 RyR fusion protein. The exact binding kinetics of the interaction could not be measured as the exact 5 concentration of the polyclonal RyR antibodies in the IgG fractions was unknown. However, using different concentrations of IgG from different patients in curve fitting models were the RyR antibody concentration was set to be 1-10% of the total IgG concentrations,  $K_D$  was 10 estimated to be about  $10^{-9}$  (data not shown). Since the Western blot conditions are also for high affinity antibodies, we conclude that the pc2 RyR antibodies are of high affinity.

The pc2 RyR antibodies inhibited cresol activated  $\text{Ca}^{2+}$  release from isolated SR fractions in a concentration dependant manner suggesting allosteric inhibition. We have previously shown that MG sera containing RyR antibodies inhibit binding of ryanodine to the RyR which also indicate that the RyR antibodies lock the RyR in the closed position. The RyR antibodies reacting with pc2 were responsible for this effect since the inhibition of  $\text{Ca}^{2+}$  release disappeared when the pc2 antibodies were removed from the IgG fractions. The pc2 region is located near a clipping site for a protease and therefore probably located 25 on the surface of the cytoplasmic part and the RyR, the foot-region. A portion of this region of the molecule is also interacting with another region on the neighbouring region of the RyR tetramer, and is therefore probably important for the conformation of the receptor (Wu, Y., B. Aghdasi, S.J. Dou, J.Z. Zhang, S.Q. Liu, and S.L. Hamilton. 30 1997, *J. Biol. Chem.* 272: 25051-25061). A potential calmodulin binding site is also located near the pc2 region. Calmodulin is very important for RyR regulation. The MG patients RyR antibodies do probably interfere with 35 RyR function; locking the receptor in the closed position; by affecting calmodulin binding or interfering with the

"self association between the subunits". By mapping the exact epitope for the MG RyR antibodies one might learn more about RyR function.

Whether antibodies against intracellular molecules are of any pathogenic significance is controversial (Alarcon-Segovia, D., A. Ruiz-Arguelles, and L. Llorente. 1996, *Immunol. Today.* 17:163-164). Antibodies are able to penetrate the cell membrane and can often be found intracellularly bound to their target antigens (not shown for the RyR antibodies). How they get there and whether they can exert their effector functions is unknown. However, this study shows that the RyR antibodies do affect RyR function directly so if they could also penetrate the cell membrane one would expect a severe effect on muscle function as the antibodies bind to the receptor with high affinity and seem to lock the channel in the closed state thus inhibiting  $\text{Ca}^{2+}$  release and muscle contraction.



SEQ ID NO 1

pc2

5 EFKFLPPPGYAPCHEAVLPRERLRLEPIKEYRREGPRGPHLVGPSRCLSHTDFVPCPV  
DTVQIVLPPHLERIREKLAENIHELWALTRIEQGWATYGPVRDDNKRJHPCLVNFHSLP  
EPERNYNLQMSGETLKTLALGCHVGMADEKAEDNEKKTKLPKTYMMMSNGYKPABLDL  
SHVRLTPAQTTLVDRLAENGHNWARDRVAQGWSYSAVQDI PARRNPRLVPYRLDEA  
TKRSNRDSLQAVRTLLGYGYNIEPPDQEPEPSQVENQSRWDRVRIFRAEKSYTQSGRW  
10 YFEFEAVTTGEMRVGWARPELRPDVELGADELAYVFNGHRGQRWHLGSEPFGRPWQSG  
DVVGCMIDL TENTIIFTLNGEVLM SD

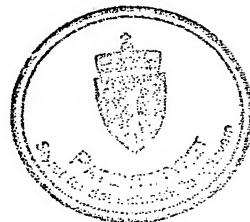
15

SEQ ID NO.2

pc25

20 RGRSLTKAQRDVIEDCLMALCRYIRPSMLQHLLRRLVEDVPLNNEFAKMPPLKLLTNHY  
ERCWKYYCLPTGWANFGVTSEELHLTRKLFWGIFDSLAHKKYDQELYRMAMPCLCAI  
AGALPPDYVDASYSSKAEKKATVDAEGNFDPBPVETLNVIIPEKLDSEINKFAEYTHE  
KWAFDKIQNNWSYGENVDEELKTHPMLRPYKTFSEKDKEIYRWPBKESLKAMIAWEWT  
IEKAREGEEERTEKKKTRKISQTAQTYDPREGYNPQPPDLSGVTLSRELOQAMAELAE  
NYHNTWGRKKQELEAKGGGTHPLLVPYDTLTAKEDREKAQELLKFQMNNGYAVT

25



## CLAIMS

1. A method for the detection of ryanodine receptor  
5 antibodies in patient serum samples, said antibodies being  
associated with the disease myasthenia gravis, said method  
comprising the following steps:

(a) obtaining a serum sample from a patient suspected  
10 of having myasthenia gravis or being at risk for the  
development of said disease;

(b) contacting said serum sample with a composition of  
fusion proteins comprising the following sequences: SEQ ID  
15 NOS 1 or 2;

c) detecting fusion protein-antibody complex  
formation, wherein said detected complexes indicate the  
presence of ryanodine receptor antibodies.

20 2. The use of the fusion proteins comprising the  
following sequences: SEQ ID NOS 1 or 2 for the detection  
of RyR antibodies.

25 3. A diagnostic kit for the detection of ryanodine  
receptor autoantibodies in patient serum samples, said  
autoantibodies being associated with the disease myasthenia  
gravis, said kit comprising fusion proteins having the  
following sequences: SEQ ID NOS 1 or 2.

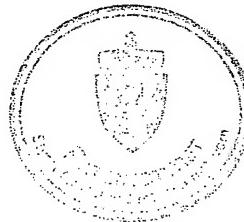
30 4. The diagnostic kit of claim 3, wherein the  
immunodetection reagent is a radiolabeled reagent.

5. The diagnostic kit of claim 3, wherein the presence of  
35 pc2 or pc25 fusion protein antibodies is indicative of a  
the presence of a thymoma

6. A composition of fusion proteins useful for the detection of ryanodine receptor antibodies, which are associated with the disease myasthenia gravis, said 5 proteins being selected from the group of proteins having of a sequence SEQ ID NO 1 or 2, or a combination of said sequences.

7. A method for the manufacture of a pharmaceutical agent 10 for the prevention and/or treatment of the disease myasthenia gravis, wherein said agent is administered to a patient in need thereof, in a amount sufficient to inhibit the binding of ryanodine receptor antibodies to the ryanodine receptor, said composition comprising a panel of 15 fusion proteins having sequences SEQ ID NOS 1 and/or 2.

8. A method of myasthenia gravis prognosis which involves the determination of the presence of RyR antibodies wherein the RyR antibodies are identified by the use of the fusion 20 proteins pc2 and pc25.



Abstract

The present invention describes methods, kits and  
5 compositions for the detection of ryanodine receptor  
antibodies in patient serum samples. The invention also  
describes a method for the manufacture of a pharmaceutical  
agent for the prevention and/or treatment of the disease  
myasthenia gravis, and a method of myasthenia gravis  
10 prognosis.

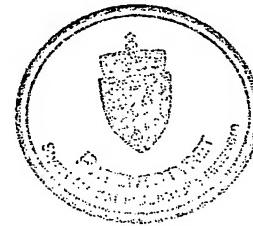


Fig. 1A

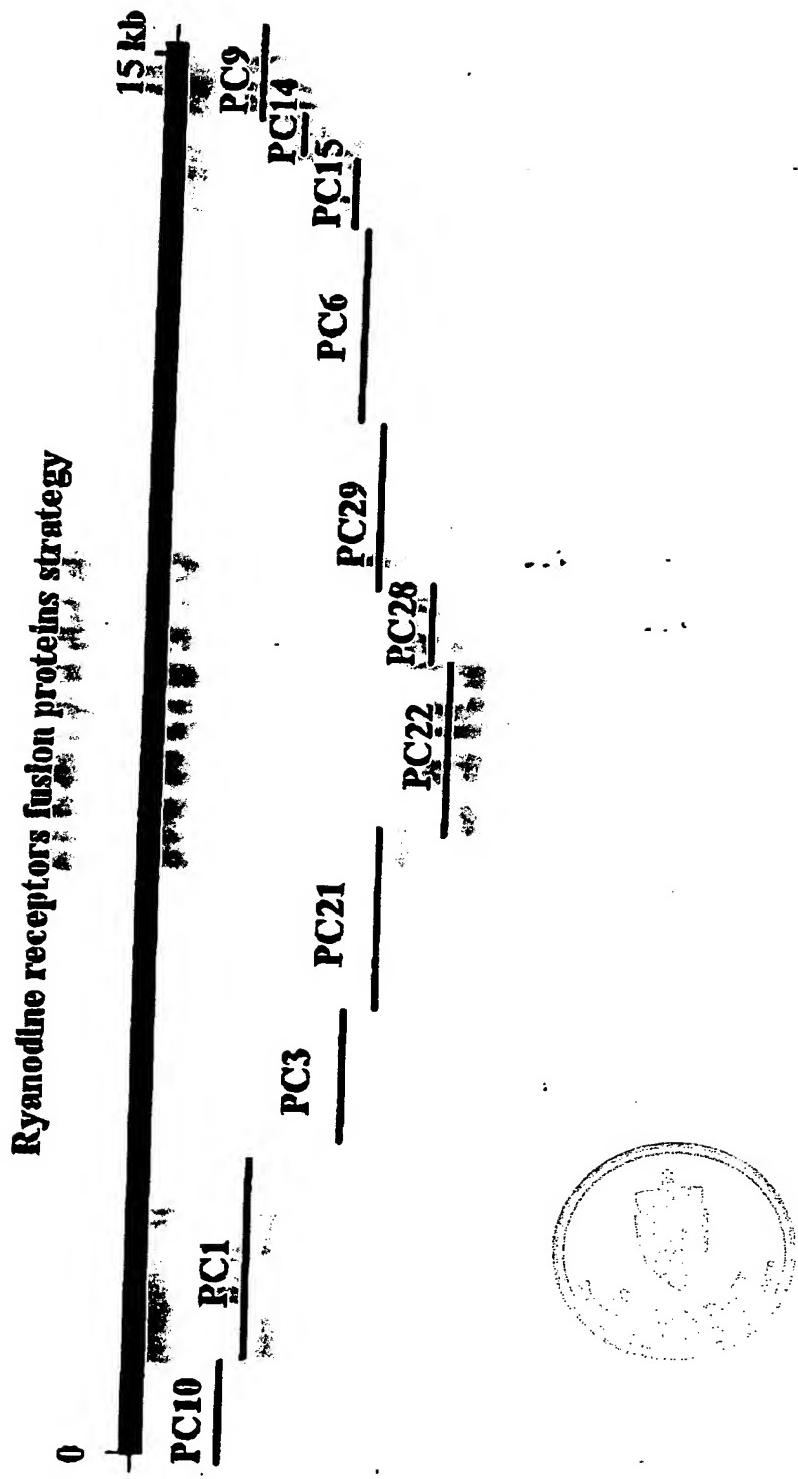
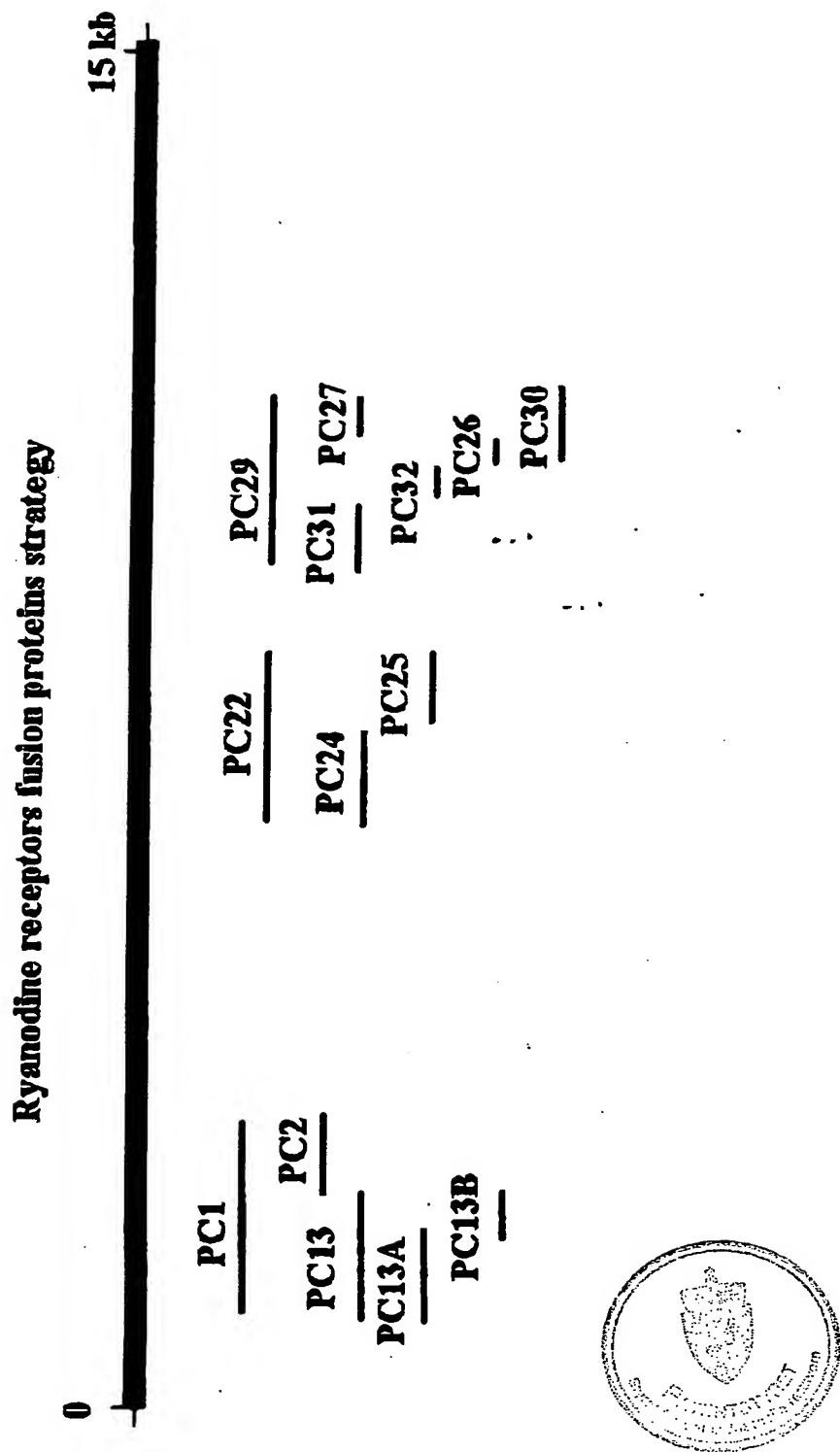


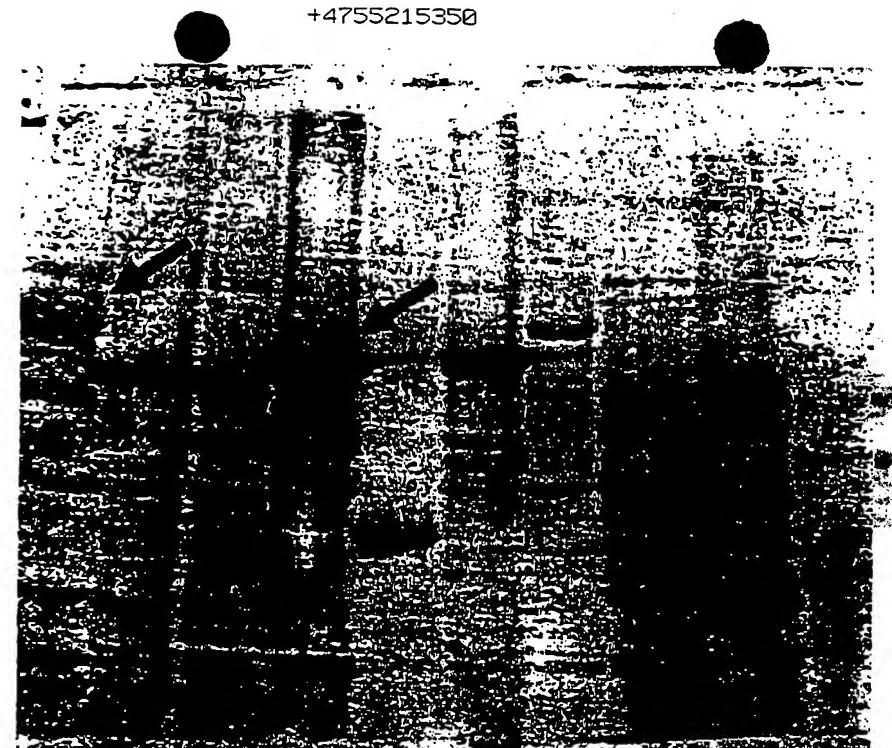
Fig. 2A



kDa

110 →

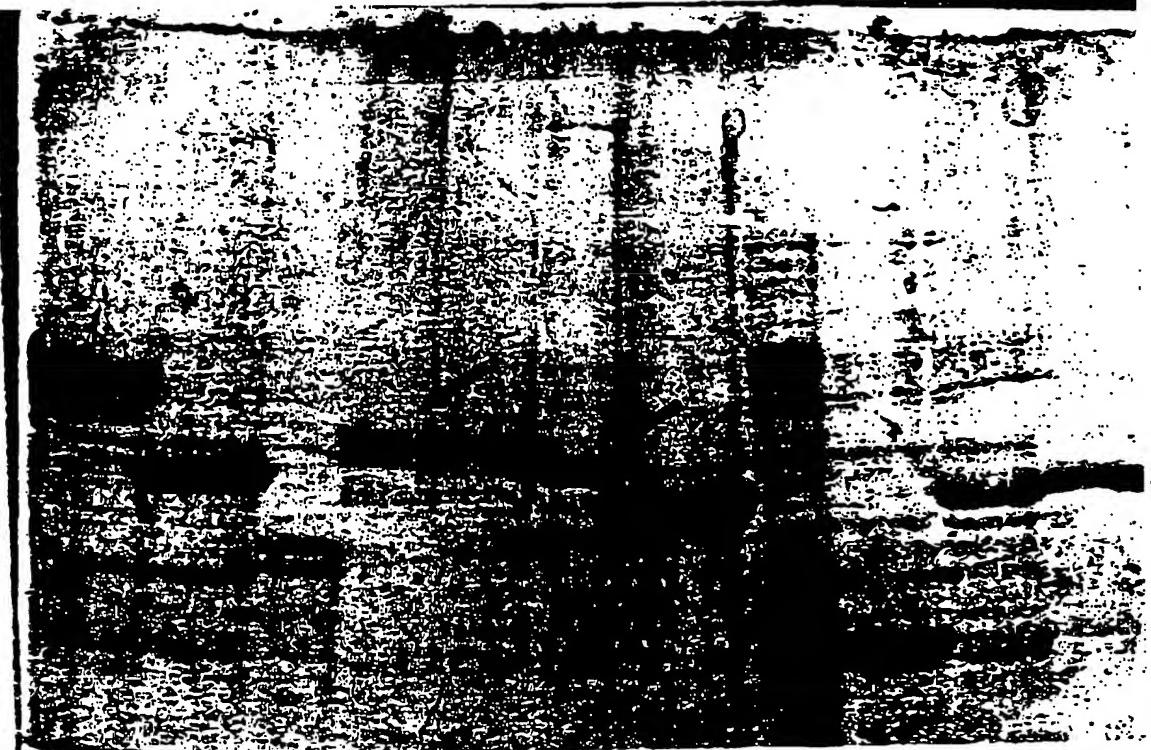
63 →



1 2 3 4 5 6 7 8 9 10

PC9  
PC14  
PC15  
PC6  
PC29  
PC28  
PC21  
PC22  
PC3  
PC1

Fig. 1B

**kDa****110 →****63 →****1 2 3 4 5 6 7 8 9 10 11**

PC13

PC13A

PC13B

PC2

PC24

PC25

PC31

PC32

PC26

PC27

PC30

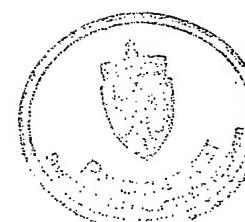
**Fig. 2B**

Fig. 3



**2      2A      2B**  
PC2    PC2A    PC2B

**2      2A      2B**  
PC2    PC2A    PC2B

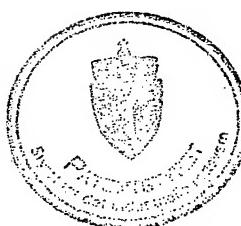
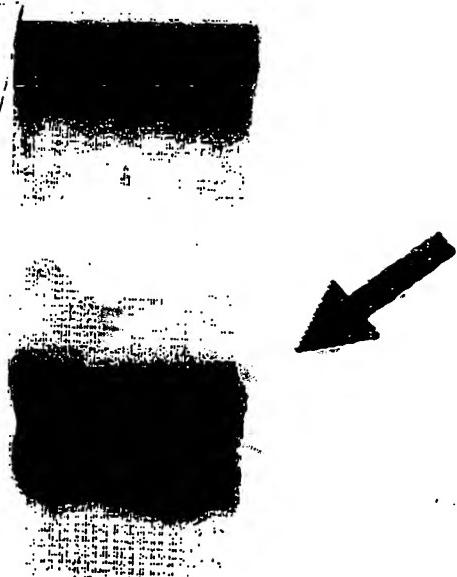


Fig. 4A

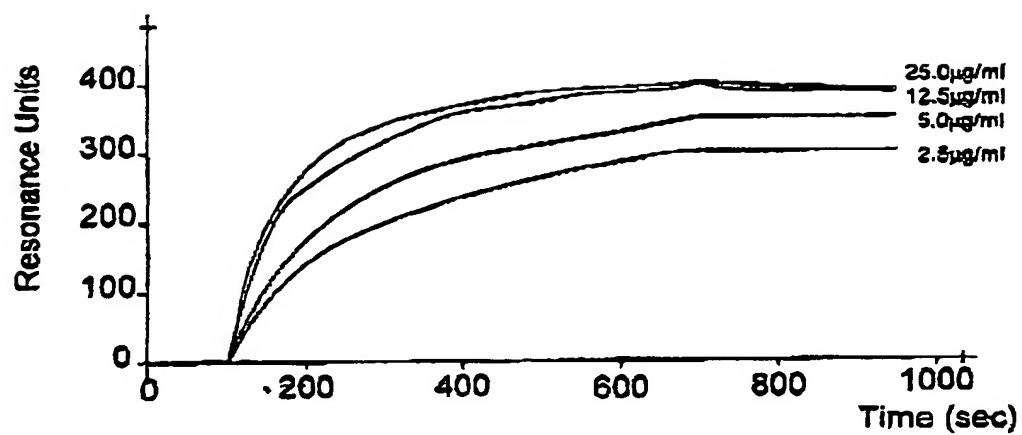


Fig. 4B

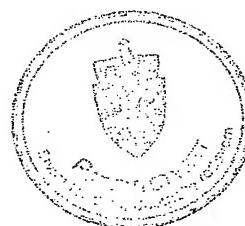
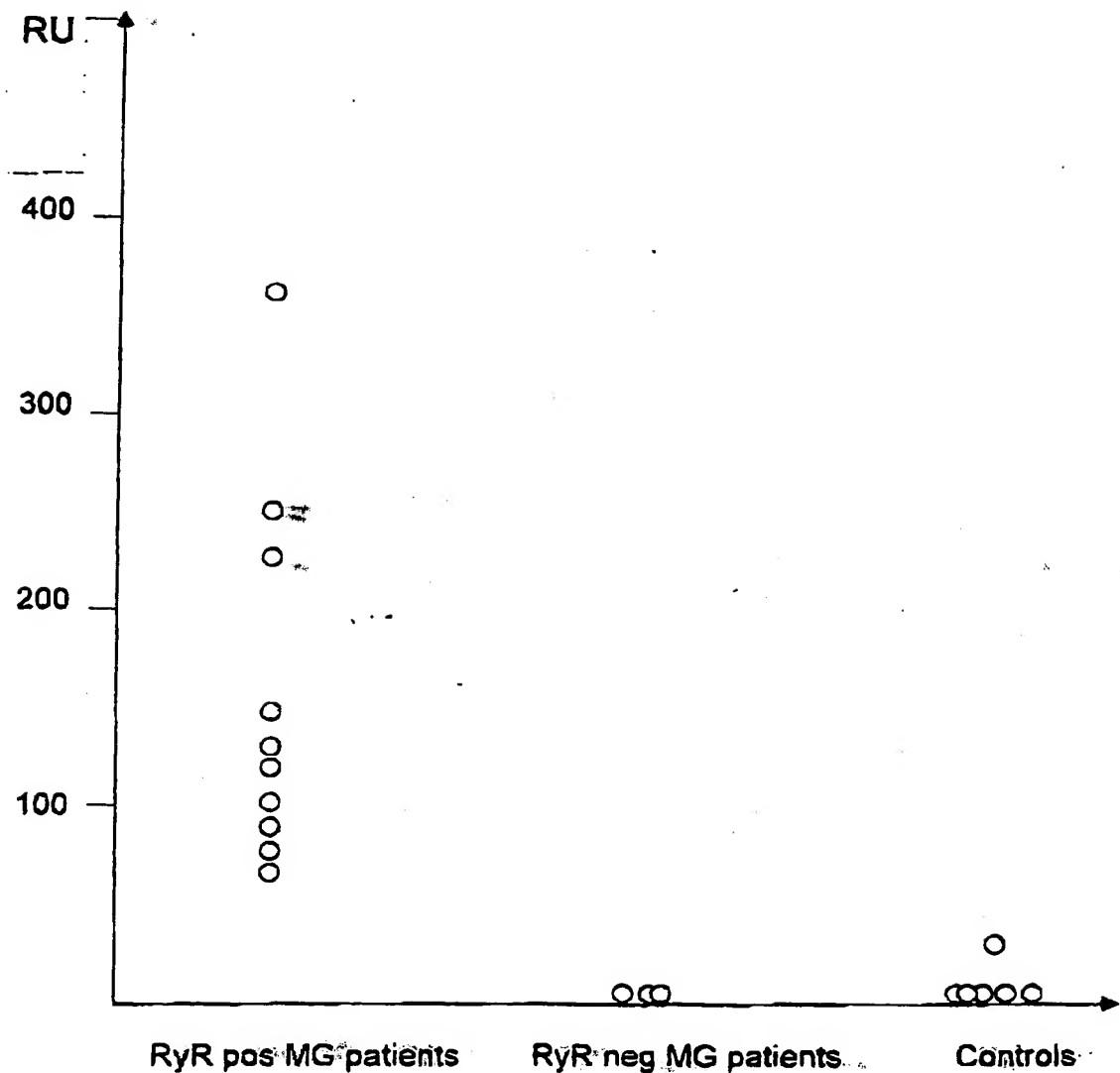


Fig. 4C

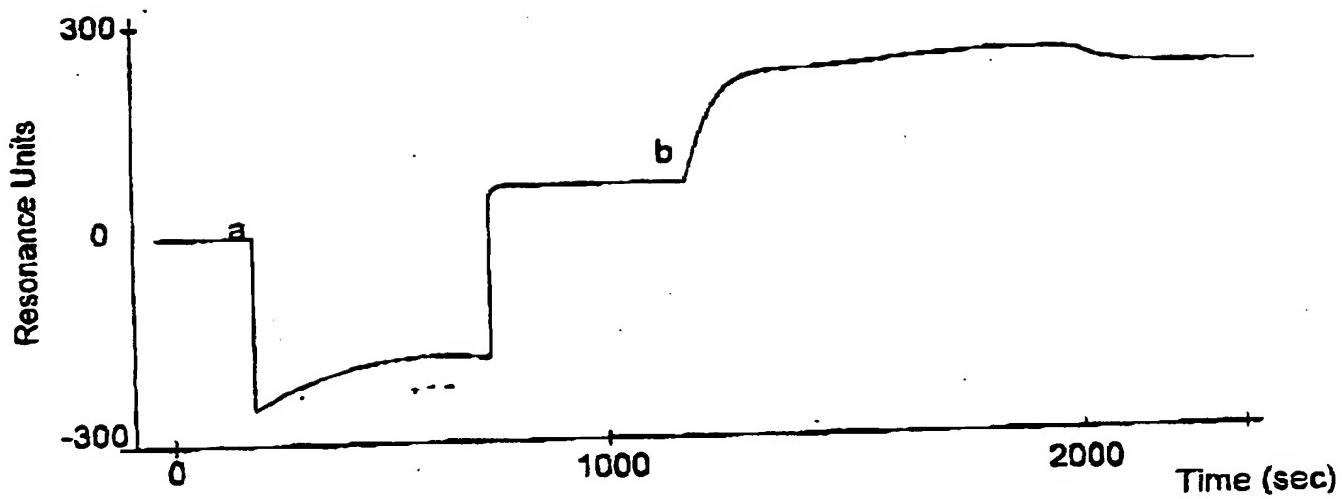
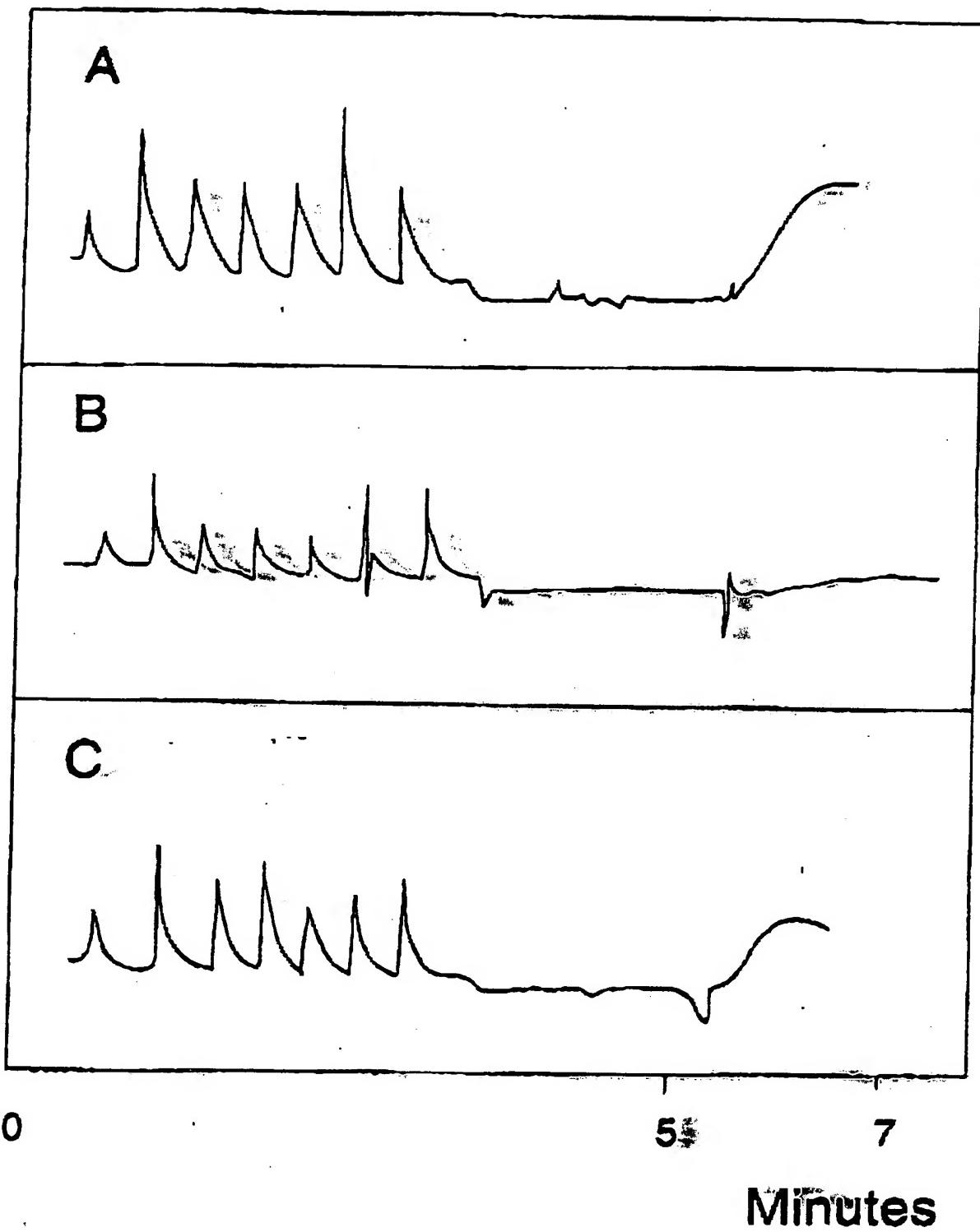


Fig. 5

A 710 - 790



Minutes

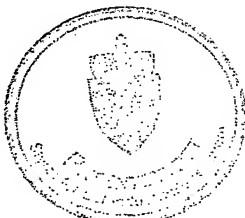
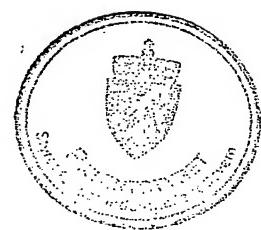
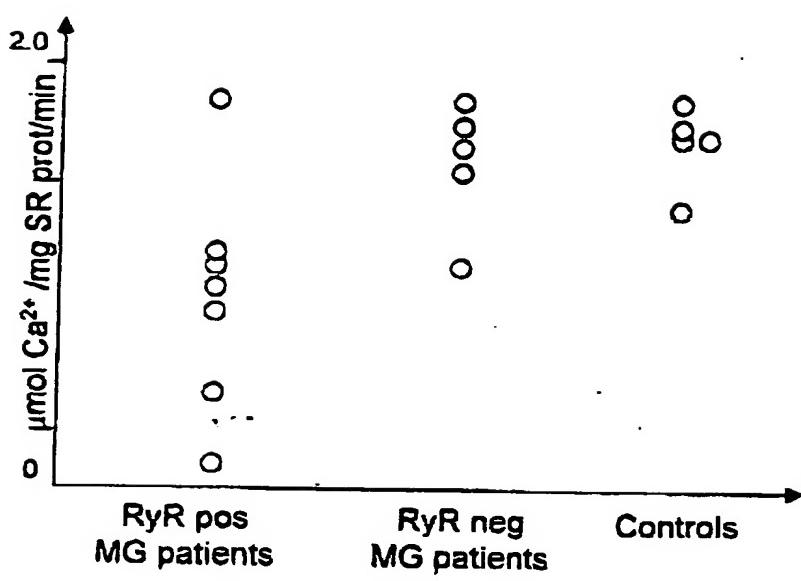


Fig. 6



**THIS PAGE BLANK (USPTO)**

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**

**THIS PAGE BLANK (USPTO)**